Genetic and biochemical mechanisms limiting fipronil toxicity in the LPR strain of house fly, *Musca domestica*

Zhimou Wen and Jeffrey G Scott*

Department of Entomology, Comstock Hall, Cornell University, Ithaca, NY 14853, USA

Abstract: Fipronil is a new insecticide which exerts its toxic action by interacting with the insect GABA-gated chloride channel. Previous studies have shown that cyclodiene-resistant insects have low to moderate levels of cross-resistance to fipronil, while other resistant strains are usually susceptible. In contrast, we recently found a strain (LPR) of house fly (*Musca domestica* L) with 15-fold cross-resistance to fipronil that was not associated with cyclodiene resistance. Fipronil cross-resistance in LPR was inherited as an intermediately dominant, autosomal, multigenic trait. [^{14}C]Fipronil was observed to penetrate into LPR flies more slowly than into susceptible flies. *S,S,S*-tributylphosphorotrithioate and diethyl maleate pretreatment did not reduce the level of fipronil cross-resistance, while piperonyl butoxide resulted in a slight decrease. These results indicate that decreased penetration and monooxygenase-mediated detoxification may be mechanisms contributing to fipronil cross-resistance in the LPR strain.

Keywords: Insecta; insecticide resistance; cytochrome P450 monooxygenases; pharmacodynamics; penetration; *Rdl*

1 INTRODUCTION

Fipronil [(±)-5-amino-1-(2,6-dichloro-α,α,α-trifluoro-*p*-tolyl)-4-trifluoromethylsulfinylpyrazole-3-carbonitrile] is a new and promising insecticide which exerts its toxic action by interacting with the insect GABA-gated chloride channel. As with any new insecticide, the potential for cross-resistance to previously (or currently) used insecticides needs careful examination. Previous studies have shown that cyclodiene-resistant *(Rdl)* German cockroaches (*Blattella germanica* (L)), fruit flies (*Drosophila melanogaster* Meig and *D simulans* (Sturtevant)) and house flies (*Musca domestica* L)[1–3] have 7.7- to 73-fold levels of cross-resistance to fipronil, while other resistant strains are usually susceptible. In contrast, we previously reported a strain of house fly (LPR) with 15-fold cross-resistance to fipronil that could not be attributed to *Rdl*.[2] The current study was undertaken to clarify the inheritance and linkage of this cross-resistance. Additionally, the mechanisms involved in the fipronil cross-resistance in LPR were examined by using [^{14}C]fipronil to study penetration rates, and synergists were used to gain information about the possible role of metabolism.

2 EXPERIMENTAL

2.1 House fly strains and crosses

CS is an insecticide-susceptible strain.[4] LPR is a multi-resistant strain having high levels of resistance to pyrethroid insecticides,[5,6] 15-fold cross-resistance to fipronil and low levels of resistance to cyclodiene insecticides.[2] The OCR strain (provided by Dr FW Plapp Jr) is highly resistant to cyclodiene insecticides and has 31-fold cross-resistance to fipronil.[2]

To determine which autosomes were associated with fipronil cross-resistance in LPR, we evaluated the toxicity of fipronil against five lines (ie strains) of flies (previously isolated by Liu and Scott[7]) which had a specific combination of autosomes from the LPR strain incorporated into the genome of the susceptible aabys strain (aabys has mutant markers on each linkage group). The phenotypes of the five lines we used were *ac*, +, +, +, +(R2345); +, *ar*, +, +, +(R1345); +, +, *bwb*, +, +(R1245); +, +, +, *ye*, +(R1235) and +, +, +, +, *sw* (R1234). These strains were named according to the autosomes lacking mutant markers (ie R2345 had autosomes 2, 3, 4 and 5 wild type (from LPR), and the mutant marker for autosome 1 (from aabys)). A detailed description

* Correspondence to: JG Scott, Department of Entomology, Comstock Hall, Cornell University, Ithaca, NY 14853-0901, USA
E-mail: JGS5@CORNELL.EDU
Contract/grant sponsor: Hatch Project; contract/grant number: 139414
Contract/grant sponsor: Rhône-Poulenc
(*Received 24 August 1998; revised version received 16 April 1999; accepted 2 June 1999*)

 Pestic Sci 0031–613X/99/$17.50

Strains	LD_{50} (ng per insect) (95% CI)	n	slope (±SE)	RR[a]
CS	7.1 (6.4–7.9)	700	3.8 (±0.4)	–
LPR	91.5 (70.9–118)	800	1.2 (±0.1)	13
OCR[b]	210 (180–250)	1500	1.9 (±0.1)	30
R2345	85.1 (78.1–92.7)	1800	3.0 (±0.2)	12
R1345	9.2 (8.5–9.9)	1880	3.7 (±0.2)	1.3
R1245	11.8 (10.8–12.7)	880	5.4 (±0.6)	1.7
R1235	20.6 (18.0–23.3)	1600	2.0 (±0.1)	2.9
R1234	10.1 (9.0–11.2)	800	3.8 (±0.4)	1.4
F_1 (CS♀ × LPR♂)	43.5 (38.0–49.5)	640	2.8 (±0.2)	6.1
F_1 (LPR♀ × CS♂)	38.0 (32.0–44.5)	640	2.2 (±0.2)	5.4
F_1 (OCR♀ × CS♂)	13.7 (12.3–15.2)	700	3.6 (±0.4)	1.9
F_1 (CS♀ × OCR♂)	11.8 (10.7–13.0)	700	3.7 (±0.3)	1.7

[a] Resistance ratio: LD_{50} of resistant strain/LD_{50} of CS.
[b] From Scott and Wen.[2]

Table 1. Toxicity of fipronil to house flies treated by topical application

of the methods used to isolate each of these strains has been previously reported.[7]

Reciprocal crosses (CS × LPR) were performed to evaluate the inheritance of fipronil cross-resistance in LPR. Fifty virgin females were collected within 8 h of emergence and caged with 50 males. Bioassays were performed on the F_1 generation as described below.

Although there are reports of cross-resistance to fipronil in cyclodiene-resistant insects, the inheritance of this cross-resistance has not been examined. To accomplish this, reciprocal crosses (CS × OCR) were performed to evaluate the inheritance of fipronil cross-resistance in a cyclodiene-resistant strain (OCR).

2.2 Topical application bioassays

Bioassays were conducted by topical application in 0.5 µl of acetone as described previously.[2] Diethyl maleate (DEM) or *S,S,S*-tributylphosphorotrithioate (DEF) were applied (10 µg per fly in 0.5 µl acetone) 1 h before fipronil. Bioassay data were pooled and analyzed based on standard probit analysis[8] as adapted to personal computer use,[9] using Abbott's correction for control mortality.[10] Fipronil (96%) was from Rhone-Poulenc (Research Triangle Park, NC), DEM (97%) was from Aldrich (Milwaukee, WI) and DEF (98%) was from Chem Service (West Chester, PA).

2.3 Penetration studies

[^{14}C]Fipronil (specific activity, 25.6 mCi mmole^{-1}, Rhone-Poulenc, Research Triangle Park, NC) was purified on HPTLC-GHLF plates (Analtech, Newark, DE) using methylene chloride as eluant. The silica gel containing [^{14}C]fipronil was extracted with acetone, resulting in [^{14}C]fipronil with a purity of >98%. The time course of fipronil penetration was studied by applying purified [^{14}C]fipronil in 0.5 µl acetone (5500 dpm, 42.3 ng) to the thoracic notum of three-to-five-day-old female CS or LPR house flies. Even though these doses of fipronil were higher than the 72-h LD_{50}, fipronil is slow-acting[2] and these doses did not adversely effect the flies during the time course of this experiment. Five treated flies were placed in a 7-ml scintillation vial. At 0, 1, 2, 4, 6, 8 and 10 h, the flies were treated as follows. Flies were transferred to a vial with 4 ml of acetone and mixed (by vortex) for about 20 s. The acetone was transferred to a scintillation vial and the files were extracted again with 4 ml of acetone. The external body rinses were combined, evaporated and analyzed by liquid scintillation counting (LSC) using a Beckman LS 5801 and Ecoscint scintillation fluid (National Diagnostics, Atlanta, GA). The washed flies were then homogenized (Teflon-glass) in 4 ml of acetone. This mixture was transferred to a scintillation vial and the homogenizer was rinsed twice with 2 ml of acetone. The homogenate and the rinses were combined, evaporated and analyzed by LSC. The holding vials were also analyzed by LSC. By this method, more than 98% of the applied radiolabel was recovered immediately after application. The experiment was repeated four times for each time point. Cpm were converted to dpm for all samples prior to analysis of the data. The penetration rate constant (K_{pen}), was calculated based on the amount of external radiolabel at the 0, 1, 2, and 4 h time points. K_{pen} was calculated assuming first-order rate kinetics,[11] which represents penetration through the insect cuticle very well,[6,12,13]

3 RESULTS AND DISCUSSION

3.1 Inheritance of fipronil cross-resistance in LPR

Fipronil cross-resistance in the LPR strain is inherited as an intermediately dominant trait (Table 1). LD_{50} values of the F_1 flies from reciprocal crosses between CS and LPR flies were not significantly different (Table 1). This suggests that cross-resistance to fipronil in LPR flies is not sex-linked nor due to cytoplasmic effects.

3.2 Autosomes associated with fipronil cross-resistance in LPR

The chromosomal linkage of fipronil cross-resistance in LPR was evaluated by bioassays using strains of

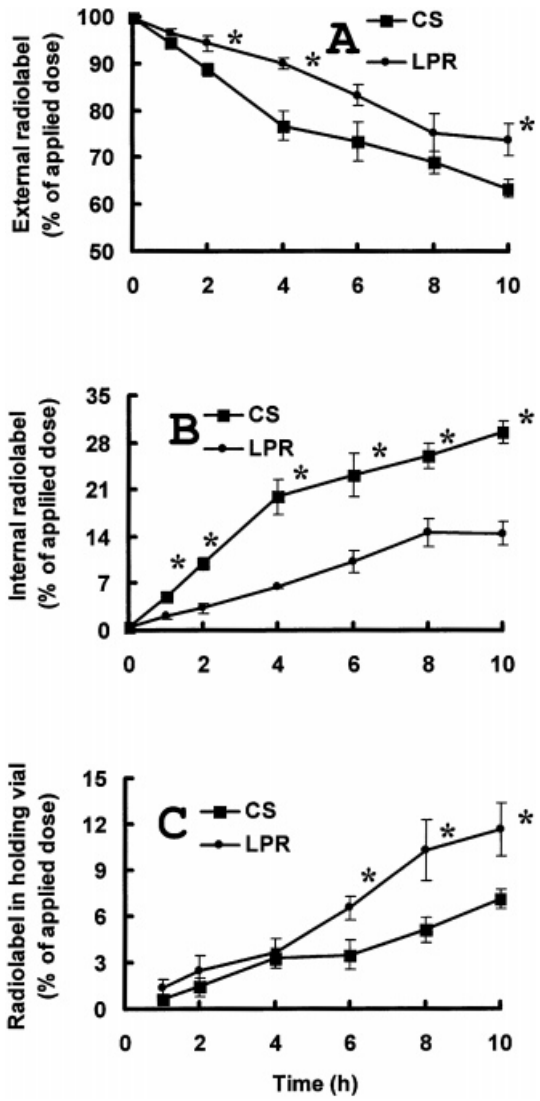

Figure 1. Pharmacodynamics of [^{14}C]fipronil penetration into CS and LPR strains of house flies. At different time points the amount of (A) external, (B) internal and (C) holding vial radiolabel was determined by LSC. Each time point represents the mean ± SE (n = 4). Significant differences between the strains ($P \leq 0.05$, determined by Student's t-test) are indicated by an asterisk.

house flies that had one autosome from LPR 'replaced' by the corresponding autosome from a susceptible strain (by a series of crosses).[7] By examining the level of fipronil cross-resistance in these strains (relative to LPR), we could identify the autosomes involved. The R2345 strain had the same level of cross-resistance as LPR (Table 1), indicating that autosome 1 was not involved. However, the other four strains (R1345, R1245, R1235 and R1234) all had LD_{50} values much lower than LPR, indicating that autosomes 2, 3, 4 and 5 were all contributing to the 13-fold fipronil cross-resistance in LPR. Thus, fipronil cross-resistance in the LPR strain appears due to minor genes on autosomes 2, 3, 4 and 5.

3.3 Penetration studies

The pharmacokinetics of [^{14}C]fipronil penetration into the CS and LPR strains of house flies are shown in Fig 1. The penetration of [^{14}C]fipronil was faster in CS than LPR, as measured by disappearance of external radiolabel (Fig 1A) or accumulation of internal radiolabel (Fig 1B). The fipronil K_{pen} values for CS and LPR were 6.65 ($\pm$1.03) $\times 10^{-2}$h^{-1} and 2.55 ($\pm$0.31) $\times 10^{-2}$h^{-1} (mean ($\pm$SE), n = 4) for CS and LPR, respectively. These results indicate that slower penetration is probably a mechanism of cross-resistance to fipronil in the LPR strain. The value of K_{pen} for [^{14}C]fipronil (6.65×10^{-2}h^{-1}) is less than that of [^{14}C]permethrin (15.8×10^{-2}h^{-1})[6] and similar to that of [^{3}H]abamectin (7.55×10^{-2}h^{-1})[13] in susceptible house flies (the [^{14}C]permethrin and [^{3}H]abamectin K_{pen} values reported here were recalculated from the previously published values). This is consistent with the toxicity of permethrin being more rapid than that of fipronil or abamectin.[6,13] The amount of radiolabel in the holding vials, representing excreted radiolabel (and possibly radiolabel that was rubbed off), was similar between strains until 6 h post-treatment; from then on the LPR vials showed higher levels. This suggests that enhanced detoxification (or possibly increased excretion of parent compound) might be involved in fipronil cross-resistance in LPR.

3.4 Effects of synergists on fipronil cross-resistance in LPR

The effects of synergists on the toxicity of fipronil to house flies are summarized in Table 2. DEM had little effect on the toxicity of fipronil in both the susceptible (CS, 1.3-fold) and resistant (LPR, 1.1-fold) strains, suggesting that glutathione transferases are not involved in the detoxification of fipronil in either strain. DEF synergized the toxicity of fipronil in both CS (5.5-fold SR) and LPR (2.4-fold SR) strains. Given that fipronil lacks an ester bond, the synergism observed does not appear due to inhibition of hydrolases. Instead, the DEF synergism may result from inhibition of monooxygenases, as has been suggested for fipronil synergism in German cockroaches.[14] PBO synergized the toxicity of fipronil 10- and 15-fold in CS and LPR, respectively,[2] indicating

Table 2. Effect of three synergists on the toxicity of fipronil to house flies

Strain	*Synergist*	*SR*[a]	*RR*[b]
CS	none	–	–
CS	DEM	1.3	–
CS	DEF	5.5	–
CS[c]	PBO	10	–
LPR	none	–	13
LPR	DEM	1.1	15
LPR	DEF	2.4	29
LPR[c]	PBO	15	9.7

[a] Synergism ratio = LD_{50} of fipronil alone/LD_{50} fipronil + synergist.
[b] Resistance ratio = LD_{50} LPR/LD_{50} susceptible CS strain.
[c] From Scott and Wen.[2]

that monooxygenase-mediated detoxification of fipronil occurred in each strain, but at a slightly higher level in LPR (Table 2).

This study has shown that cross-resistance to fipronil in the LPR strain of house fly is the result of multiple minor genes on autosomes 2, 3, 4 and 5. Results of previous genetic studies on house flies indicate that decreased penetration (*pen*) is found on autosome 3.[15] Monooxygenase-mediated resistance is usually associated with autosomes 1, 2 and/or 5.[7,15,16] The only resistance trait previously found on autosome 4 is cyclodiene resistance.[15] Therefore, it appears that fipronil cross-resistance in LPR is due to decreased penetration (autosome 3) and monooxygenase-mediated detoxification (autosomes 2 and/or 5). The minor mechanism involved in cross-resistance on autosome 4 does not appear to be *Rdl* as the level of resistance to cyclodiene insecticides in LPR is very low.[2]

CYP6D1 is the cytochrome P450 isoform responsible for monooxygenase-mediated pyrethroid resistance in the LPR strain of house fly.[17–19] Increased transcription of *CYP6D1*, which is the underlying cause of this resistance,[20] is controlled by factors on autosomes 1 and 2.[20,21] Given that autosome 1 is not involved in fipronil cross-resistance in LPR, it appears that CYP6D1 is not involved in this cross-resistance.

3.5 Inheritance of fipronil cross-resistance in OCR

Cross-resistance to fipronil in the OCR strain was inherited as an incompletely recessive trait, with the F_1 progeny having <2-fold cross-resistance to fipronil (Table 1). This is in contrast to the inheritance of cyclodiene resistance in house flies which is inherited as an intermediately dominant trait.[22,23] Although cyclodiene insecticides and fipronil are both neurotoxins that act at the GABA-gated chloride channel, their precise sites of attack on this transmembrane protein appear to be slightly different.[24] This is probably why the level of protection against cyclodiene insecticides in *Rdl* strains is so much higher than against fipronil. This may also help to explain why the inheritance of cyclodiene resistance (intermediately dominant)[22,23] and fipronil cross-resistance (incompletely recessive) are different in OCR. It has previously been shown that inheritance of resistance (or cross-resistance) due to an altered target site can be quite dissimilar between different insecticides.[25] The incompletely recessive inheritance of fipronil cross-resistance in OCR is also important because under field conditions nearly all individuals would probably be homozygous susceptible or heterozygous for *Rdl* (ie homozygous *Rdl* individuals would be extremely rare).[26] The low level of cross-resistance to fipronil in *Rdl* heterozygotes suggests that cross-resistance to fipronil, resulting from selection with cyclodienes, will pose little problem for initial control of house flies, and probably other insects, in the field.

ACKNOWLEDGEMENTS

We thank Tim Alefantis for technical assistance, G Brookhart for advice on fipronil purification, and P Korytko and C McCulloch for help with the statistical analysis. This study was supported by Hatch Project 139414 and a grant from Rhone-Poulenc.

REFERENCES

1 Colliot F, Kukorowski KA, Hawkins DW and Roberts DA, Fipronil: a new soil and foliar broad-spectrum insecticide. *Proc Brighton Crop Prot Conf – Pests and Diseases*, BCPC, Farnham, Surrey, UK. pp 29–34 (1992).

2 Scott JG and Wen Z, Toxicity of fipronil to susceptible and resistant strains of German cockroaches (Dictyoptera: Blattellidae) and house flies (Diptera: Muscidae). *J Econ Entomol* **90**:1152–1156 (1997).

3 Cole LM, Roush RT and Casida JE, *Drosophila* GABA-gated chloride channel: modified [^{3}H]EBOB binding site associated with ala-ser or gly mutants of *Rdl* subunit. *Life Sci.* **56**:757–765 (1995).

4 Scott JG, Sridhar P and Liu N, Adult specific expression and induction of cytochrome $P450_{1pr}$ in house flies. *Arch Insect Biochem Physiol* **31**:313–323 (1996).

5 Scott JG and Georghiou GP, Rapid development of high-level permethrin resistance in a field-collected strain of house fly (Diptera: Muscidae) under laboratory selection. *J Econ Entomol* **78**:316–319 (1985).

6 Scott JG and Georghiou GP, Mechanisms responsible for high levels of permethrin resistance in the house fly. *Pestic Sci* **17**:195–206 (1986).

7 Liu N and Scott JG, Genetics of resistance to pyrethroid insecticides in the house fly, *Musca domestica*. *Pestic Biochem Physiol* **52**:116–124 (1995).

8 Finney DJ, *Probit Analysis*, 3rd edn, University Press, Cambridge (1971).

9 Raymond M, Presentation d'un programme Basic d'analyse log-probit pour microordinateur. *Cah ORSTROM ser Ent med Parasitol.* **23**:117–121 (1985).

10 Abbott WS, A method of computing the effectiveness of an insecticide. *J Econ Entomol* **18**:265–267 (1925).

11 Barrow GM, *Physical Chemistry*, 3rd edn, McGraw-Hill, New York (1973).

12 Ford MG, Greenwood R and Thomas PJ, The kinetics of insecticide action. Part I: the properties of a mathematical model describing insect pharmacokinetics. *Pestic Sci* **12**:175–198 (1981).

13 Konno Y and Scott JG, Biochemistry and genetics of abamectin resistance in the house fly. *Pestic Biochem Physiol* **41**:21–28 (1991).

14 Valles SM, Koehler PG and Brenner RJ, Antagonism of fipronil toxicity by piperonyl butoxide and *S,S,S*-tributyl phosphorotrithioate in the German cockroach (Dictyoptera: Blattellidae). *J. Econ Entomol* **90**:1254–1258 (1997).

15 Tsukamoto M, Methods of genetic analysis of insecticide resistance, in *Pest Resistance to Pesticides*, ed by Georghiou GP and Saito T, Plenum Press, New York. pp 71–98 (1983).

16 Scott JG and Georghiou GP, The biochemical genetics of permethrin resistance in the Learn-PyR strain of house fly. *Biochem Genet* **24**:25–37 (1986).

17 Zhang M and Scott JG, Cytochrome b_5 is essential for cytochrome P450 6D1-mediated cypermethrin resistance in LPR house flies. *Pestic Biochem Physiol* **55**:150–156 (1996).

18 Korytko PJ and Scott JG, CYP6D1 protects thoracic ganglia of house flies from the neurotoxic insecticide cypermethrin. *Arch Insect Biochem Physiol* **37**:57–63 (1998).

19 Wheelock GD and Scott JG, The role of cytochrome $P450_{1pr}$ in deltamethrin metabolism by pyrethroid-resistant and -susceptible strains of house flies. *Pestic Biochem Physiol* **43**:67–77 (1992).

20 Liu N and Scott JG, Increased transcription of CYP6D1 causes cytochrome P450-mediated insecticide resistance in house fly. *Insect Biochem Molec Biol* **28**:531–535 (1998).
21 Liu N and Scott JG, Inheritance of CYP6D1-mediated pyrethroid resistance in house fly (Diptera: Muscidae). *J Econ Entomol* **90**:1478–1481 (1997).
22 Georghiou GP, March RB and Printy GE, A study of the genetics of dieldrin resistance in the housefly (*Musca domestica* L.) *Bull Wld Hlth Org* **29**:155–165 (1963).
23 Guneidy AM and Busvine JR, Genetical studies on dieldrin resistance in *Musca domestica* L and *Lucilia cuprina* (Wied). *Bull Entomol Res* **55**:499–507 (1964).
24 Deng Y, Palmer CJ and Casida JE, House fly head GABA-gated chloride channel: four putative insecticide binding sites differentiated by [^{3}H]EBOB and [^{35}S]TBPS. *Pestic Biochem Physiol* **47**:98–112 (1993).
25 Bourguet D and Raymond M, The molecular basis of dominance relationships: the case of some recent adaptive genes *J Evol Biol* **11**:103–122 (1998).
26 Gross PEJ and McKenzie JA, Selection, refugia, and migration: simulation of evolution of dieldrin resistance in *Lucilia cuprina* (Diptera: Calliphoridae). *J Econ Entomol* **89**:288–301 (1996).